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REMARKS

Claims 40-42 and 44-52 were pending in this application. New claims 53-61 are added. Accordingly, upon entry of this paper, claims 40-42 and 44-61 are pending and presented for consideration. Support for the new claims is found in the specification as originally filed at least at page 20, lines 8-23. Claims 40 and 46-52 are currently amended without any intent of disclaiming equivalents thereof. Applicant respectfully submits that the amendments do not introduce new matter and are made without any intention to abandon the subject matter as filed, and with the intention that claims of the same, greater, or lesser scope may be filed in a continuing application.

Oath or Declaration

The Examiner stated that the oath or declaration was missing from the Response to Office Action filed on October 10, 2003. Accordingly, Applicant submitted a Supplemental Response on February 9, 2004, containing a copy of the declaration filed in parent case 08/500, 917.

Rejections under 35 U.S.C. §112, first paragraph

The Examiner rejected claims 40-42 and 44-52 under 35 U.S.C. §112, first paragraph, alleging that Applicant did not have possession of the invention at the time of filing. In particular, the Examiner alleges that the specification does not provide any evidence that a mutation in the Factor V/V$_a$ gene is the cause of expression of APC-resistance and a predisposition to develop thrombosis, and that the claimed invention had not been reduced to practice at the time the application was filed. Applicant traverses this rejection to the extent it is maintained over the amended and new claims.

An objective standard for determining compliance with the written description requirement is whether the description clearly allows persons of ordinary skill in the art to recognize that he or she invented what is claimed. *See Amgen Inc. v. Hoechst Marion Roussel, Inc.*, 314 F.3d 1313, 1328, (Fed Cir. 2003); *Enzo Biochem, Inc. v. Gen-Probe, Inc.*, 323 F.3d 956,

969, (Fed Cir. 2002); MPEP § 2163.02. The court stated in *Amgen* that "the patentee need only describe the invention as claimed, and need not describe an unclaimed method of making the claimed product." *Amgen*, 314 F.3d at 1331. The court further recognized that "[c]ompliance with the written description requirement is essentially a fact-based inquiry that will 'necessarily vary depending on the nature of the invention claimed.'" *Enzo*, 323 F.3d at 963. Although the court held in *Fiers* and in *Eli Lilly* that the adequate description of claimed DNA requires a precise definition of the DNA sequence itself, not merely a recitation of its function or a mere wish or plan for obtaining the claimed DNA, the court in *Enzo* recently clarified that "it is not correct, however, that all functional descriptions of genetic material fail to meet the written description requirement." *Enzo*, 323 F.3d at 963; *Fiers v. Sugano*, 984 F.2d 1164 (CAFC 1993); *The Regents of the University of California v. Eli Lilly and Company*, 119 F.3d 1559 (CAFC 1997). "The written description requirement can be met by 'showing that an invention is complete by disclosure of *sufficiently detailed, relevant identifying characteristics* . . . i.e., complete or partial structure, other physical and/or chemical properties, functional characteristics when *coupled with a known or disclosed correlation between function and structure*, or some combination of such characteristics.'" *Enzo*, 323 F.3d at 963 (emphasis added) (quoting the PTO's Guidelines for Examination of Patent Applications Under the 35 U.S.C. 112, P1, "Written Description" Requirement, 66 Fed. Reg. 1099, at 1106). Specifically, the *Enzo* court held that the written description requirement may be met for generically claimed nucleotide sequences defined only by their biological activity or function, viz., the ability to hybridize to *N. gonorrhoeae* in a ratio of better than about five with respect to *N. meningitidis* "if the functional characteristic of preferential binding to *N. gonorrhoeae* over *N. meningitidis* were [sic] coupled with a disclosed correlation between that function and a structure that is sufficiently known or disclosed." *Id.* at 963-964.

Applying this standard to the instant application, Applicant submits that the specification of the instant application provides adequate written description of the <u>claimed</u> invention. First, Applicant submits that, unlike *Fiers* and *Eli Lilly*, in which DNA compositions were <u>claimed</u>, the instant application does not claim a particular mutation in the Factor V gene. The instant

application contains independent claims to (1) a method for determining if an individual has an increased risk of developing thrombosis due to inherited APC-resistance caused by a gene mutation (claim 40), (2) a method for determining an increased risk of developing thrombosis in an individual (claim 46 and claim 47), (3) a method for determining an increased risk of APC-resistance in an individual (claim 48), and (4) a method for identifying an occurrence of a Factor V gene mutation associated with APC-resistance in an individual (claim 54). Applicant submits that the specification provides written description for the claimed methods and need not describe an unclaimed mutation in the Factor V gene.

Second, contrary to the Examiner's statement on page 4 of the Office Action that the specification does not provide any evidence that there is a mutation in the Factor V/V$_a$ gene that causes APC-resistance, Applicant submits that a clear correlation between APC-resistance and the Factor V gene is described throughout the specification. In particular, on page 20, lines 10-11, the specification discloses that there is a strong linkage between a known polymorphism in the Factor V gene and expression of APC-resistance. It was well known in the art at the time the instant application was filed that certain DNA polymorphisms are indicative of the presence of a disease gene. Zbar, The Biology and Genetics of Hereditary Cancers, Seminars in Oncology Nursing, 8(4):229-234, (1992), a copy of which is enclosed as Exhibit A. "Minor DNA sequence differences allow the preparation of remarkably powerful analytic reagents. These reagents enable research workers to determine [the] location of disease genes precisely, which in turn permits presymptomatic diagnosis and disease gene isolation." Id. at 233 (Exhibit A). "Once linkage between a DNA marker of known chromosomal location and an inherited disease has been clearly identified, the focus turns to identification of the disease gene, and a number of strategies become possible." Keating, Linkage Analysis and Long QT Syndrome: Using Genetics to Study Cardiovascular Disease, Circulation, 85:1973-1986, (1992), a copy of which is enclosed as Exhibit B. In the instant case, the strong linkage between the polymorphism in the Factor V gene and the expression of APC-resistance, coupled with a disclosed functional correlation between the Factor V gene and the APC-resistance, allows a person of ordinary skill in the art to readily recognize that a mutation in the Factor V gene is responsible for the APC-

resistance. Thus, as set forth on page 20, lines 12-23, one skilled in the art would readily be able to determine an occurrence of a mutation in the Factor V gene that causes APC-resistance by analyzing the nucleotide sequence using nucleic acid hybridization, nucleic acid sequencing, and other methods known in the art, and by comparing the nucleotide sequence of the Factor V gene from the individual with APC-resistance to the nucleotide sequence of a Factor V gene from a normal individual, which was known in the art when the application was filed.

Although the precise mutation in the Factor V gene that causes APC-resistance is not disclosed, Applicant submits that the disclosure of the precise mutation is not required for practicing the claimed inventions. First, Applicant submits that the mutation or mutations in the Factor V gene that cause APC-resistance are readily ascertainable by the methods described on page 20, lines 8-15, and claimed in claim 54. A person of ordinary skill in the art knows that only deviations from the normal sequence will have to be considered. As set forth on page 20, lines 15-19, such deviations may include the abnormal presence or absence of one or more nucleic acid fragments, abnormal sequences, or combinations thereof. Second, it is possible that more than one type of mutation in the Factor V gene may cause APC-resistance in an individual, making it difficult, if not impossible, to include in the specification every possible mutation in the Factor V gene that may cause APC-resistance.

Applicant further submits that amended claims 40, 46, 47, and 48 recite methods for determining an increased risk of developing thrombosis or APC-resistance in an individual. Accordingly, Applicant submits that the description of a precise mutation is not necessary to practice the inventions as claimed in claims 40, 46, 47, and 48. "Once genetic markers have been identified that are linked to the disease gene it is possible to predict the risk of an asymptomatic individual being a disease gene carrier. . . . If a genetic marker has been identified that is coinherited with the disease gene, it is possible to predict risk with greater precision." Zbar, at 233 (Exhibit A). The specification of the instant application discloses that there is a strong linkage between a known neutral polymorphism in the Factor V gene and the phenotype of APC-resistance. Thus, this correlation indicates that an individual that has the polymorphism also has

a mutant Factor V gene and therefore has an <u>increased risk</u> of being a disease gene carrier and an <u>increased risk</u> of developing thrombosis or APC-resistance.

Applicant therefore submits that the specification of the instant application fully complies with the written description requirement by showing that the claimed inventions are complete by disclosure of *sufficiently detailed, relevant identifying characteristics, i.e.,* a strong linkage between a polymorphism in the Factor V gene and expression of APC-resistance, *coupled with the disclosed functional correlation* between the Factor V gene and expression of APC-resistance as is required by the current case law and the PTO's Guidelines for Examination of Patent Applications Under the 35 U.S.C. 112, P1, "Written Description" Requirement. Accordingly, Applicant respectfully requests that this rejection be reconsidered and withdrawn.

REQUEST FOR TELEPHONIC INTERVIEW

The undersigned respectfully requests a telephonic interview with Examiner Ethan Whisenant in order to expedite the favorable prosecution of the case. The Examiner is invited to telephone Diana Steel at 617-310-8168 to arrange a convenient time to discuss any outstanding issue, and to work with the Examiner toward placing the application in condition for allowance.

CONCLUSION

Applicant respectfully urges that all claims are in condition for allowance and requests prompt and favorable action on the instant application.

Respectfully submitted,

Date: April 9, 2004
Reg. No. 43,153

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The Biology and Genetics of Hereditary Cancers

Berton Zbar

THERE is considerable interest and excitement being generated among workers researching the inherited forms of human cancer. Newly developed tools offer the opportunity of gaining a deeper understanding of rare tumors and their common counterparts. These new tools enable research workers to locate the defective genes responsible for the inherited forms of cancer (Fig 1). Once a disease gene has been localized, it may then be possible to identify individuals who have inherited the disease gene before cancer develops. Identification of gene carriers, then, may lead to early diagnosis and treatment of cancer. The purpose of this article is to describe the basic principles of the biology and genetics of hereditary cancers.

DISTINGUISHING HEREDITARY FROM NONHEREDITARY (SPORADIC) CANCERS

Cancer is a common disease. Many families have more than one member who have had cancer; this usually does not represent hereditary cancer. Table 1 compares sporadic and inherited cancers. In most families with more than one individual affected with cancer, the affected individuals are over 50 years of age and have only one cancer of a common type (eg, carcinoma of the colon, lung, breast, or pancreas). Inherited forms of cancer occur in younger individuals; tumors are multiple and may involve both members of a paired organ (Fig 2). In hereditary cancers, two or more first-degree relatives (parent, child, or sibling) frequently have the same type of cancer. A pedigree of a family with inherited cancer is shown in Figure 3. This family, a family with von Hippel-Lindau disease, contains six affected individuals. The mother (individual no. 2), her four daughters, and one grandchild are known to have one or more of the manifestations of von Hippel-Lindau disease. Individual no. 18, the sister of individual no. 2, is not affected, nor are any of her children or grandchildren. The disease gene is transmitted to the descendants of individual no. 2, but not to the descendants of individual no. 18. In the absence of a positive family history, it is still possible that an individual has hereditary cancer. If an individual develops multiple tumors at a young age, it is possible that this individual has inherited a new germline mutation.

Families with a hereditary cancer syndrome have a predisposition to develop specific types of cancer. For example, families with familial polyposis are predisposed to develop adenomas and carcinomas of the colon, but not carcinomas of the kidney, lung, or breast. Families with von Hippel-Lindau disease have a predisposition to develop multiple renal cell carcinomas, but not carcinomas of the colon, lung, or breast.

DETECTION AND DIAGNOSIS OF HEREDITARY CANCERS

How can physicians and nurses detect new families with hereditary cancer? The criteria that should lead the medical professional to suspect the possibility of hereditary cancer are as follows: (1) the development of a cancer in an individual who is younger than usual for that histologic type of tumor, (2) the development of multiple tumors of the same histologic type, (3) a family history of cancer, and (4) the development of certain unusual types of tumors (eg, hemangioblastoma of the cerebellum, medullary carcinoma of the thyroid, neurofibroma, pheochromocytoma, retinoblastoma, and Wilms' tumor). It is important to note that family members may not have had the same manifestation of the illness and still be affected. In von Hippel-Lindau disease the disease manifestations include tumors of the eye, brain, spinal cord, and kidney. An affected family member may have any one of these manifestations.

HOW RESEARCH IS CONDUCTED IN INHERITED FORMS OF HUMAN CANCER

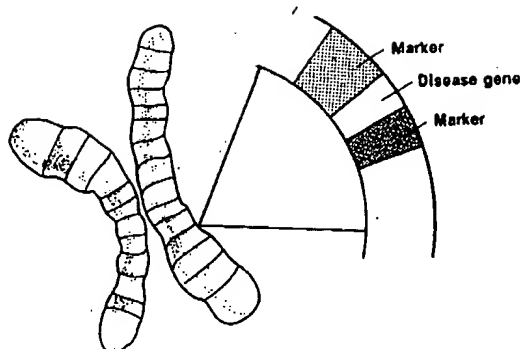Families with inherited forms of cancer may be identified by looking for case reports in the litera-

Fig 1. Locating a disease gene. The elongated, worm-like structures are chromosomes. A small region has been magnified to show a disease gene flanked by genetic markers. Genetic markers are characteristics that may be inherited in association with the disease. These markers assist in localization of the disease gene. (Copyright 1987 by the New York Times Company. Reprinted by permission.)

ture, by referrals from families known to have the illness, by contacting medical specialists who treat the condition, and by contacting support organizations like the National Organization for Rare Disease (PO Box 8923, New Fairfield, CT 06812). In our studies of von Hippel-Lindau disease we identified families by all these methods. Once families with the inherited illness are identified, a member of the research team contacts the family to determine if the family is willing to participate in a genetic study of the illness in their family. The genetic study involves determining which family members are affected and which family members are thought to be unaffected. Copies of medical records are obtained to substantiate the diagnosis. Death certificates, pathology reports, and summaries of hospital records are important in substantiating the diagnosis. Blood samples are obtained from each family member (both affected and not affected) for DNA analysis and for preparation of long-term B-lymphocyte cell lines. It is desirable to establish a clinical program to examine unaffected members of families with the inherited ill-

Table 1. Characteristics of Sporadic and Inherited Forms of Human Cancer

Characteristic	Sporadic	Inherited
Age of onset	Older	Younger
No. of tumors	Single	Multiple
Involvement of paired organs	One	Both
Affected parent, sibling, or child	Absent	Present*

*Except with a new germline mutation.

ness to identify asymptomatic individuals who carry the disease gene.

OUR GENETIC INHERITANCE AND HOW IT IS TRANSMITTED

We inherit from our parents approximately 50 to 100,000 genes. These genes determine our physical characteristics: for example, the color of our eyes, skin, and hair; height; facial appearance; and our susceptibility to disease. A gene is a specific sequence of DNA containing a set of instructions coding for the production of a protein. Genes are located on 46 chromosomes in the nuclei of our cells. At the time of fertilization, the sperm carries one half of our genetic endowment (23 chromosomes); the egg carries the other half (23 chromosomes). The sex of the individual is determined by the X and Y chromosomes. An individual that receives a Y chromosome from his father and an X chromosome from his mother is male. An individual that receives an X chromosome from her father and an X chromosome from her mother is female. The final 46 chromosomes (23 pairs) contain two copies of each gene: one copy inherited from each parent. The exception is the XY pair of chromosomes in which there are genes on the X chromosome that are not matched by a copy of the Y chromosome.

PATTERNS OF INHERITANCE

It is possible to derive a considerable amount of genetic information simply by observing the pattern of inheritance of a characteristic in a family. This characteristic can be an unusually shaped finger or it can be a disease. What proportion of the children are affected? Are both male and female children affected? Is the affected parent just as likely to be male as female? Once these simple questions are answered, one is well on the way to
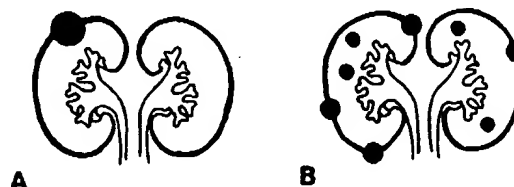

Fig 2. Sporadic (A) compared with hereditary renal cell (B) carcinoma. The solid circles indicate individual renal cell carcinomas. Sporadic cancers are usually solitary, whereas hereditary cancers are frequently multiple.
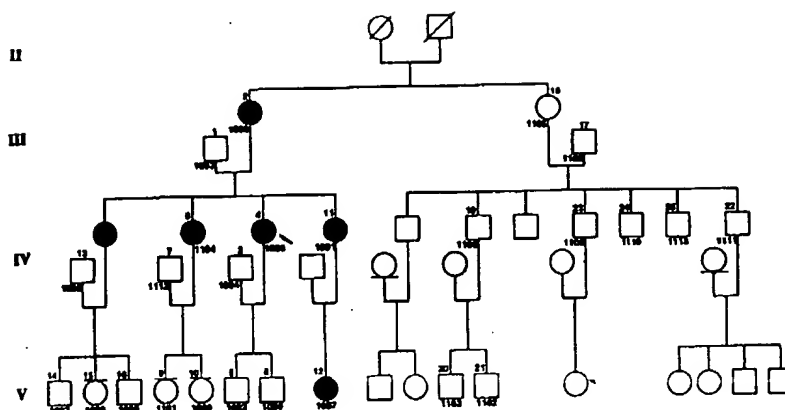
Fig 3. A family from Louisiana with an inherited form of cancer (von Hippel-Lindau disease). Circles indicate females, squares indicate males, solid symbols indicate affected individuals, and open symbols indicate individuals that are not affected. The diagonal line indicates a deceased individual. See text for discussion.

understanding the mode of inheritance of the disease and having information about location of the disease gene.[1,2]

Sex-Linked Inheritance

Consider the case of an illness that occurs only in males and is inherited only from mothers (Fig 4). These characteristics identify a trait that is sex-linked (only males have the trait) and recessive (the trait is detected when the gene that determines the normal counterpart is absent). These biologic characteristics indicate that the gene responsible for the trait must be located on the X chromosome. Examples of a sex-linked recessive trait are Duchenne type muscular dystrophy and hemophilia.

Autosomal Recessive Inheritance

Consider the case of an illness that occurs both in males and females in which one parent is affected and in which there may be a history of consanguinity (mating between two relatives) (Fig 5). These are the characteristics of autosomal recessive inheritance. This gene is not located on a sex chromosome. The child must receive the mutant gene from both parents to be affected; that is, both genes in a gene pair must be affected for the disease to be manifested. Neither parent manifests the
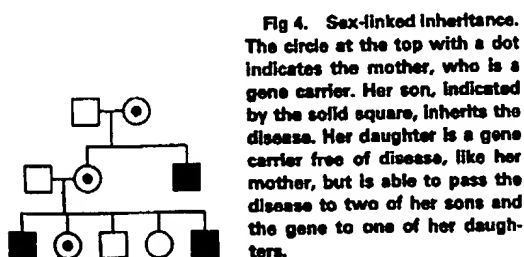

Fig 4. Sex-linked Inheritance. The circle at the top with a dot indicates the mother, who is a gene carrier. Her son, indicated by the solid square, inherits the disease. Her daughter is a gene carrier free of disease, like her mother, but is able to pass the disease to two of her sons and the gene to one of her daughters.

disease. Cystic fibrosis and xeroderma pigmentosum are examples of genetic diseases with autosomal recessive inheritance.

Autosomal Dominant Inheritance

Consider the case of an illness that occurs in both males and females in which one parent is affected and there is no history of consanguinity (Fig 3). These are the characteristics of autosomal dominant inheritance. The gene is located on an autosome (not a sex chromosome). Dominance refers to the fact that the disease in manifested when only one gene of a gene pair is abnormal. Most inherited human cancers are inherited as autosomal dominant traits.

KNUDSON'S TWO-MUTATION THEORY OF CANCER FORMATION

In a hereditary cancer, at the time of fertilization the zygote (the fertilized egg) receives an imperfect copy of a gene (a mutation). Each cell that forms from the fertilized egg will also receive this mutant gene. For reasons that are not understood, in spite of the fact that each cell in the individual's body has received one copy of the mutant gene, tumors are formed only in one or two organs and, in these organs, from only a few cells. Such observations and the observation that hereditary tumors occur earlier in life than their sporadic counterparts led Alfred Knudson to propose the two-
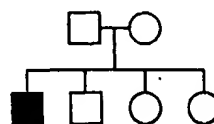

Fig 5. Autosomal recessive inheritance. Neither parent manifests the disease. A son has the disease and is presumed to have inherited a recessive gene from each parent.

mutation theory of cancer (Fig 6). This theory states that two mutations are required for tumor development to occur; these mutations inactivate both copies of a gene and so eliminate the normal protein product of the gene.

In hereditary cancers, the individual inherits one mutant gene. Inactivation of the normal counterpart of the mutant gene occurs by chance, as a result of a mitotic error, during the many cell divisions that are necessary for the formation of the individual from the fertilized egg. In sporadic cancers, both mutations occur after fertilization. One of the major simplifying postulates of the two-mutation theory is that the sporadic and hereditary forms of a cancer are caused by inactivation of the same genes; the biologic differences between sporadic and hereditary cancers reflect the differences in the time when these genes were inactivated: one mutation is inherited and the other is acquired.

TUMOR SUPPRESSOR GENES

The genes for most hereditary forms of cancer are considered to be tumor suppressor genes. These genes are called tumor suppressor genes because the normal function of these genes is to suppress cell growth and this function was first observed in relation to tumors. Inactivation of both copies of these genes leads to uncontrolled cell growth. In hereditary cancer, a tumor suppressor gene is mutated and there is a subsequent loss in
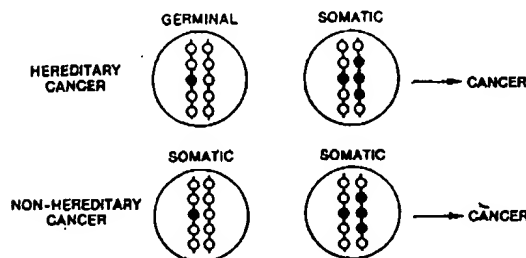

Fig 6. Knudson's two-mutation theory of cancer. Each cell contains a pair of homologous chromosomes containing the cancer suppressor gene. Solid circles indicate sites of mutation. The first mutation usually is a structural change in a specific cancer gene (single solid circle in the left chain of each pair). The second mutation usually involves damage to a much larger region of the chromosome by errors at the time of cell division (multiple solid circles in the right chains of each pair). In the hereditary cancers the initial mutation (in the left chain of each pair) is germinal (present in the germ cell) and in the nonhereditary cancers the mutations are somatic (acquired due to damage to a somatic cell). (Reprinted with permission.[3])

the tumor tissue of the normal counterpart of the gene. The concept of tumor suppressor genes received considerable support by the demonstration that introducing a normal copy of the retinoblastoma gene into retinoblastoma tumor cells reversed the ability of the cell line to form tumors. The tissue specificity of tumor suppressor genes is noteworthy. Even though these genes may be expressed in many different cell types, the types of tumors that develop when one of these genes is inactivated are remarkably specific.

GENE CARRIERS, PENETRANCE, EXPRESSIVITY, AND HETEROGENEITY

Hereditary cancer syndromes are produced by the inheritance of a single mutant gene. Several terms describe some of the biologic characteristics of inherited cancers. Consider a family in which a paternal grandfather and grandson have retinoblastoma but the father of the child does not have the eye tumor. We would say that the father of the child must be a *gene carrier* since he is the individual who is likely to have transferred the mutant gene to the child. We would say that the retinoblastoma gene was not *penetrant* in this individual. Penetrance refers to the proportion of individuals who have the mutant gene that show manifestations of the mutant gene. Penetrance varies considerably in the different forms of inherited cancer. For hereditary retinoblastoma and for von Hippel-Lindau disease, penetrance of the disease gene is 90% or greater. For Wilms' tumor, penetrance is less than 50%. *Expressivity* refers to the frequency with which all disease manifestations are apparent in individuals with the disease gene. In neurofibromatosis type 1, expressivity varies widely. Some carriers of the NF1 gene just have pigmented skin spots (cafe au lait spots) while other gene carriers have pigmented skin lesions as well as hundreds of neurofibromas.

Genetic heterogeneity means that the same clinical picture can be produced by different mutant genes, that is, genes at different chromosomal locations. If genetic heterogeneity is observed in a particular form of inherited cancer, the task of DNA-based predictive testing becomes more complicated. Genetic heterogeneity is observed in tuberous sclerosis. *Clinical heterogeneity* means that the same mutant gene can produce different clinical pictures. Clinical heterogeneity is observed in

von Hippel-Lindau disease and in familial polyposis.

TOOLS FOR STUDYING THE INHERITANCE OF DISEASE GENES: DNA POLYMORPHISMS

Minor differences in the sequence of our DNA can be detected using new tools. Minor DNA sequence differences allow the preparation of remarkably powerful analytic reagents. These reagents enable research workers to determine location of disease genes precisely, which in turn permits presymptomatic diagnosis and disease gene isolation. The term "polymorphism" means many different shapes or sizes and refers to strands of DNA of different sizes. DNA of different-sized fragments is produced by digestion with special enzymes that cut the strand of DNA at a specific location (Fig 7). Since the location is based on the precise sequence of the DNA code, a slight change in that code will produce a large change in the size of the fragments of DNA produced by enzyme treatment. To detect a particular polymorphism, one needs DNA from an individual, appropriate enzymes to cut the DNA, and a DNA fragment (a probe) that can be used to detect the particular variation in fragment size that is being searched for.

DETERMINING GENE LOCATION: LINKAGE ANALYSIS AND RISK PREDICTION

To be able to predict the risk of inheritance of a disease gene, it is necessary to identify polymor-
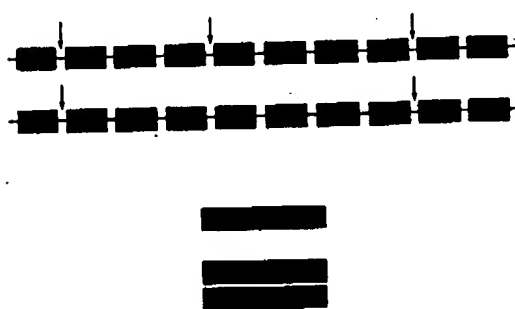

Fig 7. DNA polymorphism. The arrows indicate the site of cleavage of the DNA strand by a particular enzyme specific for that site (a restriction endonuclease). Because of a slight difference in the DNA code in the two chromosomes, the two strands of DNA are cut at different places. This leads to DNA of different sizes. The size of DNA fragments is detected by Southern transfer, hybridization, and autoradiography, as shown in the lower part of the figure. (Reprinted with permission.[5])

phic markers that are inherited along with the disease gene and to determine the frequency of recombination between these markers and the disease gene. Figure 8 depicts the concept of coinheritance or linkage.[4] The pedigree of an imaginary three-generation family has been typed with a polymorphic probe whose alleles are designated "1" and "2." There is concordance between inheritance of the disease gene and allele 2 in 12 of 13 individuals. The inheritance of the disease and this genetic locus is said to be linked. Recombination explains the observation in the one individual in whom allele 2 was not inherited with the disease gene.

Figure 9 illustrates the results in a hypothetical family in which there is independent inheritance of a disease and a genetic marker. No linkage is seen. Results of linkage analysis are expressed as recombination frequencies. A recombination frequency of 1% means that there was one recombination in 100 opportunities for recombinations (meiosis). The recombination frequency in the example in Fig 8 is $1/13 = 0.076$; the recombination frequency in the example in Fig 9 is $6/13 = 0.46$.

Once genetic markers have been identified that are linked to the disease gene it is possible to predict the risk of an asymptomatic individual being a disease gene carrier. The risk of the individual inheriting the disease gene from its affected parent is 0.5. If a genetic marker has been identified that is coinherited with the disease gene, it is possible to predict risk with greater precision.

CONCLUSIONS

Research in the genetics of hereditary cancers is in a state of ferment. Investigators around the
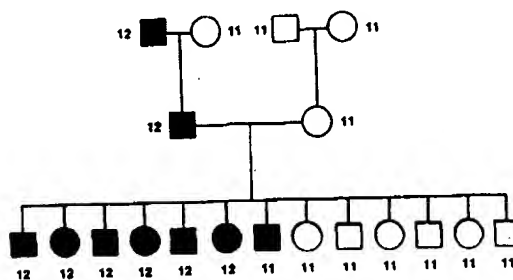

Fig 8. Coinheritance of a disease gene and a genetic marker. Shown here is an imaginary pedigree with two genes, "1" and "2," that are inherited and with a disease that is inherited (shown by the solid circles or squares). Note that in all but one case allele 2 is inherited along with the disease. This is called "linkage."
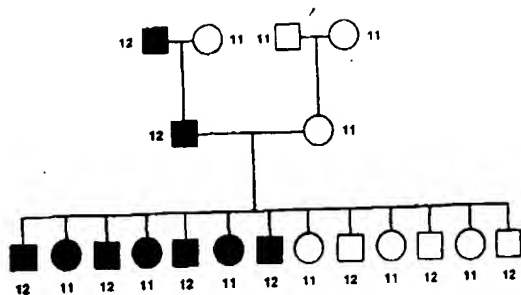
Fig 9. Independent assortment of a disease gene and a genetic marker. Note that the family members with the disease, the solid circles and squares, do not inherit the same combination of genes "1" and "2." The disease and the genetic markers are said to not be linked.

world are determining the location of genes responsible for causing inherited forms of cancer and isolating the disease genes. Progress varies in the different hereditary cancers. The disease genes have been located and cloned for retinoblastoma, neurofibromatosis type 1, Li-Fraumeni syndrome, Wilms' tumor, and familiar polyposis. For other hereditary diseases, such as von Hippel-Lindau[5,6] and neurofibromatosis type 2, the disease genes have been located and intensive cloning effects are under way. Considerable effort is being expended to locate the disease genes responsible for familial malignant melanoma, nevoid basal cell carcinoma, and breast carcinoma. Eventually, we will isolate the genes responsible for each inherited form of cancer and have DNA-based predictive testing for each familiar disorder. The isolation of the defective genes will initiate a new phase of research into the neoplasms and provide a more fundamental understanding of the nature of cellular growth control, eventually leading to better methods of cancer control and treatment.

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Focus on Molecular Biology

Linkage Analysis and Long QT Syndrome
Using Genetics to Study Cardiovascular Disease

Mark Keating, MD

Background. Recombinant DNA technologies have facilitated the development of a set of polymorphic DNA markers covering the human genome. General linkage analysis in families predisposed to inherited disease is now feasible. Linkage analysis can help identify a disease gene even when relatively little is known about the disorder.

Methods and Results. Using this approach, we have identified linkage between a gene that causes the long QT syndrome and DNA markers on chromosome 11.

Conclusions. The identification of the chromosomal location of the long QT locus is the first step in defining the specific mutations that cause this disease. (*Circulation* 1992;85:1973–1986)

KEY WORDS • DNA markers • restriction fragment length polymorphisms • recombination, genetic • arrhythmias

To discover genes that play an important role in cardiovascular disease, investigators have studied proteins that might be important in a disorder. If biochemical or physiological abnormalities were found, these investigators could work back to the gene that encoded the protein, thereby identifying the genetic mutations that caused the disorder. This approach has helped to advance our understanding of cardiovascular biology and disease. In particular, investigators have used this approach to make great progress in our understanding of abnormalities in lipoprotein and lipid metabolism.[1] Recently, investigators have taken a different and complementary approach to these problems, one that involves molecular genetics. Often referred to as "reverse genetics" or "positional cloning," this strategy begins with the chromosomal localization of a gene that is responsible for an inherited disorder. (Neither of these terms accurately describes the actual process of mapping a disease gene and ultimately identifying disease-causing mutations. The term "reverse genetics" does not reflect the process but rather the change in experimental direction from starting with proteins to beginning with genes. The term "positional cloning" is more accurate in that it implies the cloning of a gene on the basis of its chromosomal location. Merely cloning a gene located near a disease locus, however, does not prove that it is the disease gene. We prefer to use the term "mapping and mutational analysis" to describe the process of disease gene

identification outlined here.) The ultimate goal of the approach is to define abnormalities in the protein product of the gene and explain the disease pathogenesis. The purpose of this article is to review the theory and practice of the first step in this genetic strategy, linkage analysis of families with inherited disease.

Genetic Basis of Linkage Analysis

In the late 19th century, Gregor Mendel demonstrated that traits were inherited as independent units; that is, the inheritance of one trait did not influence the likelihood of inheriting a second.[2] This fundamental rule of inheritance is known as Mendel's law of independent assortment. In the early 1900's, Thomas Hunt Morgan and his coworkers proposed the existence of an exception to this law. In breeding experiments performed with *Drosophila*, these investigators noted that certain traits, or genes, were inherited together (coinherited) more frequently than would be predicted by chance.[3,4] These coinherited genes were said to be linked. It later became apparent that genes were coinherited or linked because they were physically located on the same chromosome.[5] Linkage analysis is a technique that can be used to identify genes that are coinherited and therefore are located on the same chromosomal segment.

Human cells contain 23 pairs of homologous chromosomes, for a total of 46 diploid chromosomes. An individual inherits one set of chromosomes from his mother and the other set from his father. Each chromosome consists of a linear array of double-stranded deoxyribonucleic acid (DNA); the entire genome consists of approximately 3 billion base pairs of DNA. Some of this DNA is organized as genes (approximately 100,000 of them) that encode proteins, but most of the DNA has no known function. Each chromosome contains a different complement of genes and intervening sequences. For example, the genes that encode the human lymphocyte antigens (HLAs) are always located
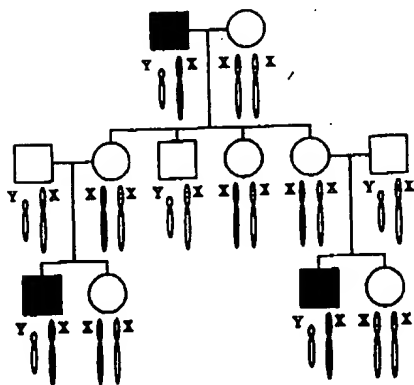
FIGURE 1.   *Map of sex-linked inheritance. A disorder caused by the inheritance of a recessive gene located on the X chromosome will affect only male offspring of carrier mothers because males carry only one copy of the X chromosome. Individuals affected by a X-linked disorder are indicated by filled squares (males). Phenotypically normal individuals are indicated by open circles (females) or open squares (males). The X chromosome carrying the recessive disease gene is filled. Females who carry the disease gene are phenotypically normal because they have a second X chromosome.*

on the short arm of chromosome 6 (chromosome 6p), whereas the gene for apolipoprotein B is always located on the short arm of chromosome 2 (chromosome 2p). The order of genes in the human genome is invariant, and the relative position of one gene is said to be its locus. Homologous chromosomes are similar but not identical. Each cell contains two copies, or alleles, of the apolipoprotein B gene, each located on its respective homologue of chromosome 2. DNA sequences that separate genes, however, may vary considerably, and even allelic forms of the same gene contain subtle DNA sequence differences.[6] The DNA sequence differences between homologous chromosomes are what make linkage analysis possible.

A well-known example of linkage in human families is sex or X-linked inheritance (Figure 1). An inherited disease that affects only sons of apparently unaffected mothers is caused by the inheritance of a recessive gene located on the sex-determining X chromosome. Sex linkage is relatively easy to identify as a mode of inheritance because of an obvious variation in the human genome; males inherit one X chromosome (from their mothers), whereas females inherit two (one from each parent). A mother with the recessive gene carries another, presumably normal, copy of that gene on her second X chromosome, so she would be phenotypically normal. Her sons, however, have a 50% likelihood of inheriting the X chromosome containing the aberrant gene and manifesting the disease phenotype.

Because the identification of X linkage was relatively simple, much of the early progress using linkage analysis involved genes located on the sex chromosomes. An example of an X-linked gene that affects the cardiovascular system is dystrophin, the gene responsible for both Duchenne and Becker muscular dystrophies.[7,8] Most inherited disorders, however, are not linked to the X chromosomes but are caused by genes located on one of the 22 pairs of homologous chromosomes (the auto-

somes). To identify linkage with genes located on the autosomes, it is necessary to identify genetic variations that are specific for those autosomes. Identifiable genetic variations are called polymorphic markers.

DNA-Based Markers

The ideal genetic marker would be easily detectable and would exist in a number of different, or polymorphic, forms. Linkage between a marker and a disease gene is detectable only if the individual with the disease gene carries two different forms of that marker. Like genes, a marker has a specific chromosomal location or locus, and different forms of the same marker are also referred to as alleles. Thus, allelic forms of the same marker share the same chromosomal location. When an individual carries two different alleles of one marker locus, he is said to be heterozygous for that marker. If the alleles are identical, he is said to be homozygous.

Until recently, only a limited number of markers were available. Most of these were protein polymorphisms like blood group antigens or HLAs. Only about 30 of these protein polymorphisms were useful, and these represented only a few chromosomes and an even smaller portion of the human genome. For lack of genetic markers, most human inherited disorders could not be approached by use of linkage analysis.

The situation improved dramatically with the advent of recombinant DNA technology in the 1970s. In 1978, the University of Utah sponsored a genetics retreat at a ski resort in the Wasatch mountains. At that meeting, David Botstein, Ronald Davis, and Mark Skolnick first suggested that variations in DNA sequence, which were known to exist in other organisms, might also exist in humans. They further suggested that these variations could be detected by use of the tools of molecular biology and that they could be used as polymorphic markers in linkage studies.[9] One year later, Kan and Dozy[10] demonstrated the existence of DNA sequence variation at the β-globin locus associated with sickle cell anemia. Shortly thereafter, DNA variation was also observed at the same locus in association with thalassemias.[11] Thus, the β-globin gene was the first DNA-based polymorphic marker. In 1980, Ray White and his colleagues identified the first highly polymorphic locus in an arbitrary, noncoding stretch of DNA (Wyman and White[12]).

Restriction Fragment Length Polymorphisms

The new strategy has proved to be very powerful. Human DNA sequences contain a high level of variation; for example, differences in sequence exist between homologous chromosomes at every 200–300 base pairs. The initial problem was how to detect them. One way to detect sequence polymorphisms is with restriction enzymes. A restriction enzyme, produced by and purified from certain bacteria, recognizes a specific sequence in a linear piece of double-stranded DNA called a restriction site, where it cleaves the DNA. Variations in DNA sequence that create or eliminate a restriction site will result in a change in the length of the resultant restriction fragment (Figure 2A). These sequence variations became known as restriction fragment length polymorphisms (RFLPs).

To detect an RFLP, the restriction fragments are first separated by agarose gel electrophoresis (Figure 3). DNA molecules are drawn through a gel by an electric field and separated on the basis of size. Over the same period, smaller fragments travel farther than larger ones. These double-stranded DNA fragments are then denatured (converted from the double-stranded to the single-stranded form), and the resultant single-stranded molecules are transferred to a nylon or a nitrocellulose membrane, a technique known as Southern blotting.[13] Once denatured, these single-stranded pieces of DNA will bind to (hybridize with) a complementary strand of DNA with great specificity.

Human DNA contains multiple copies of any given restriction site, so the filter generated by Southern blotting will contain multiple DNA fragments from all 46 chromosomes. Most of these DNA fragments will not be polymorphic. To identify the RFLP of interest, a radiolabeled piece of DNA (probe DNA) is incubated with the filter. Probe DNA must be complementary to sequences adjacent to the specific restriction site polymorphism so that it will bind to specific sequences on the filter. If the filter is then exposed to x-ray film (a process known as autoradiography), the size of the complementary DNA fragments will be revealed.

Many RFLP's are caused by a single base pair change, resulting in the presence or absence of the restriction site. The RFLP, therefore, exists in only two forms. Because each individual has two copies of the RFLP, three different combinations are possible (Figure 2A); the individual may be homozygous for one form of the RFLP (a genotype of 1,1), homozygous for the second form (a genotype of 2,2), or heterozygous (a genotype of 1,2). With luck, such a polymorphic marker may be informative for linkage in a large, well-characterized family. If a polymorphic marker and the disease locus are located on the same chromosome, one would expect the disease phenotype to be coinherited (to cosegregate) with one of the polymorphic alleles more than 50% of the time. If, on the other hand, the DNA marker and the disease gene were on different chromosomes, then one would expect them to be coinherited approximately 50% of the time, or to segregate independently. In Figure 4A, individuals thought to be affected by an inherited disorder are indicated by filled circles or filled squares. The genotypes for these individuals are also shown by an RFLP marker that shows two different alleles. The affected father in generation 1 is heterozygous at this hypothetical marker locus (a genotype of 1,2), so his children should be informative for linkage; that is, each chromosome is marked by a different allele. Each child should inherit either the 1 or the 2 allele from the affected father. In this example, all affected children inherit the 1 allele, and all nonaffected children inherit the 2 allele. If this pattern persists in subsequent generations, one can conclude that the polymorphic marker and the disease gene are likely to be located on the same chromosomal segment, or linked.

Although very useful, RFLP markers may have reduced power because of inadequate heterozygosity in the population. If, for example, the affected father in generation 1 of the pedigree in Figure 4 had a homozygous genotype (a genotype of 1,1) at this marker locus, all of his children would be expected to inherit one copy

of that allele (Figure 4B). In this example, both affected and normal children would inherit one copy of the 1 allele, and the marker would be uninformative for linkage.

Markers for Variable Number of Tandem Repeats

Fortunately, another kind of DNA marker exists, one that detects many different polymorphic forms in the general population. Human DNA contains sequences that are tandemly repeated throughout the genome (Figure 2B; References 14 and 15). The function of these tandem repeats is uncertain, but the number of repeats is highly variable, and these variations can be detected. The vast majority of these sequence variations are stably inherited (that is, they do not mutate with high frequency), so they can be used for linkage analysis. Probes for markers based on the variable number of tandem repeats (VNTRs) will detect a large number of variations in the general population, greatly increasing the likelihood that individuals in a family will be heterozygous, thereby increasing the informativeness of the marker for linkage. Like RFLPs, VNTRs can be detected by use of restriction enzymes in a technique similar to the one described above (Figure 3).
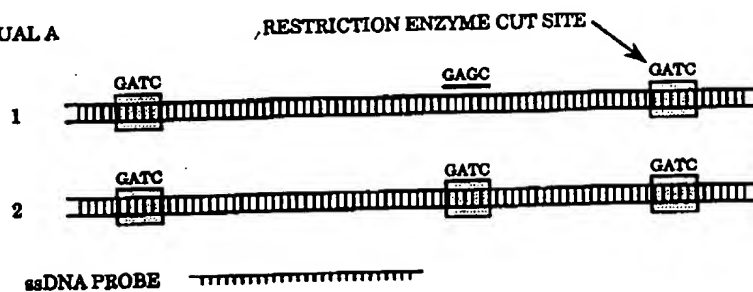
Polymerase Chain Reaction–Based Markers

Recently, a number of different types of repeated units have been identified in human genomic sequences (Figure 2C; References 16–18). As with VNTRs, the function of these repeated sequences is uncertain, but they can be used for linkage analyses if the polymorphic forms can be detected. In general, however, these repeated sequences are too small (for example, two base-pair, or dinucleotide, repeats like CA repeats) to be detected by a strategy that uses restriction enzymes. However, these small repeated sequences can be detected by use of a recent technical advance, the polymerase chain reaction (PCR; Figure 5). In PCR, a double-stranded piece of DNA is denatured and then enzymatically copied, or amplified. After one round of amplification, the result is a net doubling of DNA. If the process is repeated through multiple cycles of amplification, multiple copies of DNA will result. Thus, PCR is an enzymatic DNA copying machine.
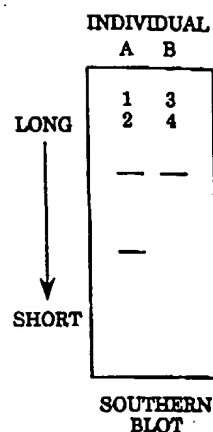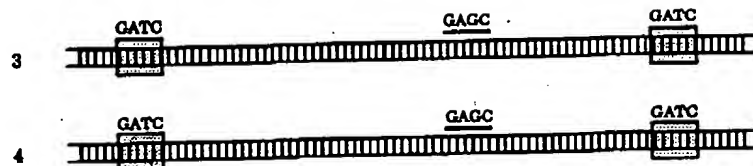
PCR specificity is in part dependent on the starting material, or template DNA. In the experiments described here, template DNA is usually human genomic DNA that contains two double-stranded copies of every locus in the genome. A second critical factor for PCR specificity results from the sequence of small synthetic pieces of DNA of approximately 20 nucleotides called oligonucleotide primers. Like probe DNA, a primer is designed to be complementary to a specific DNA sequence in the template DNA. In general, two different primers (a primer pair) that flank a specific locus of interest are used in each PCR reaction. When the template and primer pair are denatured and then cooled, the primers will bind to complementary sequences on the template. The enzyme, or polymerase, that copies DNA in PCR reactions is primer-dependent; that is, the polymerase will copy only the specific sequences that are adjacent to the primer. Furthermore, the polymerase is unidirectional; that is, it will copy adjacent sequences only from the 5' to the 3' direction.
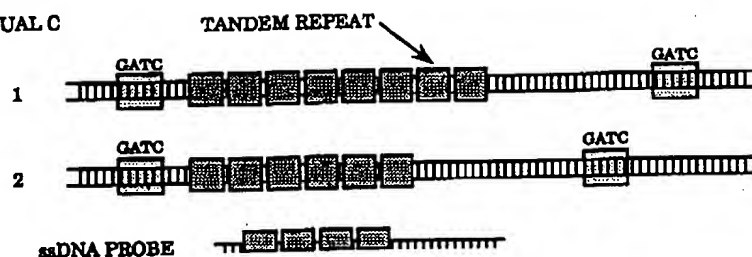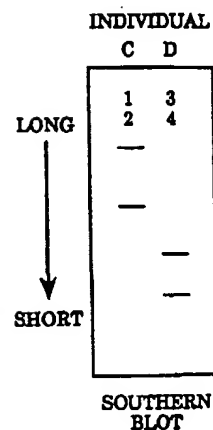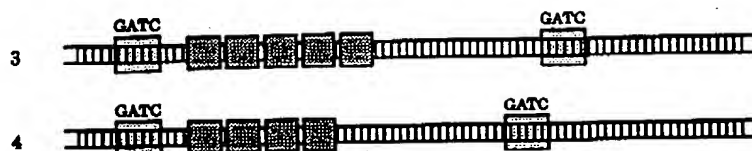
A. RFLP

INDIVIDUAL A

RESTRICTION ENZYME CUT SITE

GATC   GAGC   GATC

1

GATC   GATC   GATC

2

ssDNA PROBE

INDIVIDUAL B

GATC   GAGC   GATC

3

GATC   GAGC   GATC

4

INDIVIDUAL
A   B

1   3
2   4

LONG

SHORT

SOUTHERN
BLOT

B. VNTR

INDIVIDUAL C

TANDEM REPEAT

GATC   GATC

1

GATC   GATC

2

ssDNA PROBE

INDIVIDUAL D

GATC   GATC

3

GATC   GATC

4

INDIVIDUAL
C   D

1   3
2   4

LONG

SHORT

SOUTHERN
BLOT

C. Sequence-based polymorphisms

INDIVIDUAL E

DINUCLEOTIDE REPEAT

FOWARD PRIMER   CA CA CA CA CA CA CA CA

1

REVERSE PRIMER

FOWARD PRIMER   CA CA CA CA CA

2

REVERSE PRIMER

INDIVIDUAL F

FOWARD PRIMER   CA CA CA CA CA CA CA CA CA

3

REVERSE PRIMER

FOWARD PRIMER   CA CA CA CA CA CA

4

REVERSE PRIMER

INDIVIDUAL
E   F

1   3
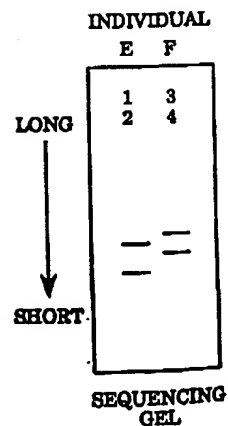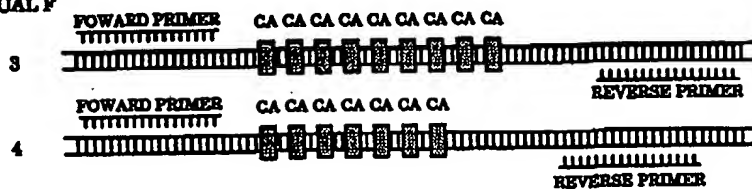2   4

LONG

SHORT

SEQUENCING
GEL
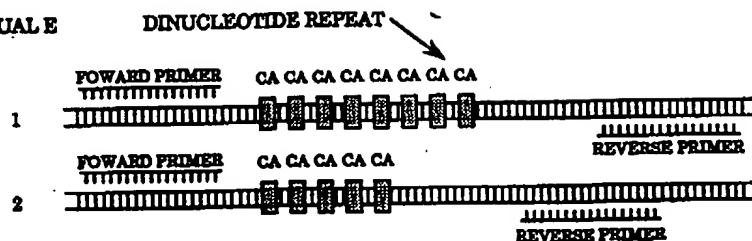
FIGURE 2. *Facing page: Schematic of DNA markers. When homologous chromosomes differ by the presence or absence of a restriction site, as shown in panel A, a restriction site polymorphism exists. Homologous chromosomes in individual A are numbered 1 and 2, whereas those in individual B are numbered 3 and 4. In individual A, a single base-pair substitution (T to G) in one homologous chromosome (chromosome 1) results in the loss of a restriction site. The restriction enzyme cuts DNA at the specific sequences indicated by a stippled box. DNA fragments with a different length will result (restriction fragment length polymorphisms [RFLPs]). By use of a single-stranded DNA probe that is complementary to sequences near the restriction site polymorphism, RFLPs separated by electrophoresis can be detected (top right). Southern blotting reveals a distinct band for each chromosome. One of three different potential patterns exists in each individual; an individual can be homozygous for one allele, homozygous for the second allele, or heterozygous. Individual A is heterozygous for this RFLP, so two bands are identified. By contrast, the restriction fragments generated from individual B's chromosomes (chromosomes 3 and 4) are identical, so only one band is seen. Another type of polymorphism is shown in individuals C and D (panel B). This type of polymorphism results from the variable number of tandem repeats (VNTRs, small, tandemly repeated sequences) that exist in homologous chromosomes. After the DNA is cut by a restriction enzyme with sites near the VNTR and then separated by electrophoresis, the polymorphisms can be detected by use of a probe with DNA complementary to sequences adjacent to the VNTR. VNTR markers are highly polymorphic and show multiple alleles in the population. A third type of polymorphism is based on sequence variations that are smaller than VNTRs and do not cause RFLPs. In individuals E and F (panel C), a variable number of dinucleotide CA repeats are shown at one locus. These polymorphisms can be detected by use of specific oligonucleotide primers in polymerase chain reactions (PCRs). The resultant PCR products will vary in size, and these size differences can be detected by acrylamide gel electrophoresis. Like VNTR markers, sequence-based polymorphisms may be highly polymorphic in the population.*

In this way, the specific sequences that separate the primer pair can be amplified from a heterogeneous population of template DNA. If the template DNA used in the reaction is human genomic DNA, each locus is represented twice, and both will be copied. If the two loci are identical, the PCR products will also be identical and, therefore, indistinguishable. If, however, the amplified locus contains a sequence-based polymorphism (sequence-tagged site, or STS) and if the individual to be tested is heterozygous for that polymorphic locus, both copies will be amplified, resulting in two PCR products of slightly different size (Figure 2C). These products can be distinguished by acrylamide gel electrophoresis, a technique that is very sensitive and can distinguish between DNA fragments differing in size by only one or two nucleotides. If the oligonucleotides used in the PCR reaction are radiolabeled, the polymorphic products can again be visualized by autoradiography.
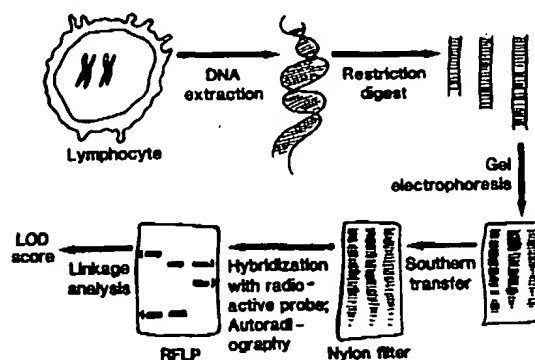
GENOTYPING

FIGURE 3. *Genotypic analysis. The process of determining the genotype of an individual by use of DNA markers that detect restriction fragment length polymorphisms (RFLPs) is outlined. Each vertical lane on the DNA filter represents one individual, and each individual has two alleles for a given DNA marker (in this case a variable number of tandem repeats marker). The RFLPs shown here are for a hypothetical mother at left (genotype of 1,4), father at right (genotype of 2,3), and their child (genotype of 2,4). Note that the child inherits one allele from each parent.*

PCR can also be used to identify nonrepetitive sequence-based polymorphisms like single base pair substitutions, deletions, or insertions. Two commonly used techniques that can detect single base pair variations are PCR–single-strand conformation polymorphism analysis (PCR-SSCP; References 19 and 20) and PCR–denaturing gradient gel electrophoresis (PCR-DGGE; Reference 21). The first step in both techniques is to amplify a small (approximately 400–base pair) section of DNA from an individual's genomic DNA. If that individual has two different versions of the DNA segment of interest, PCR will amplify both, and two different amplification products will result. These two different products can then be distinguished by SSCP or DGGE. The principle underlying SSCP is that during electrophoresis, a single strand of DNA will migrate through a nondenaturing gel at a rate that depends on the size and the specific sequence of the strand. Another strand of the same size containing a single nucleotide substitution or other sequence polymorphism will, under certain electrophoretic conditions, travel through the same gel at a slightly different rate, presumably because of conformation differences. In DGGE, by contrast, double-stranded DNA fragments are separated by electrophoresis through a gel that contains an increasing gradient of denaturant. As the DNA passes through the gel, it eventually reaches a concentration of denaturant that will convert the DNA from the double-stranded to the single-stranded form, dramatically changing the mobility of the fragments. The point at which the fragment of double-stranded DNA begins to denature depends in part on the sequence, and a single base pair substitution may make a significant difference. If the PCR products are radiolabeled, separated by SSCP or DGGE, and subjected to autoradiography, different polymorphic forms of homologous segments of DNA can be identified and used as DNA markers for

A.

Allele 1

Allele 2

Southern Blot

B.

Allele 1

Allele 2

Southern Blot
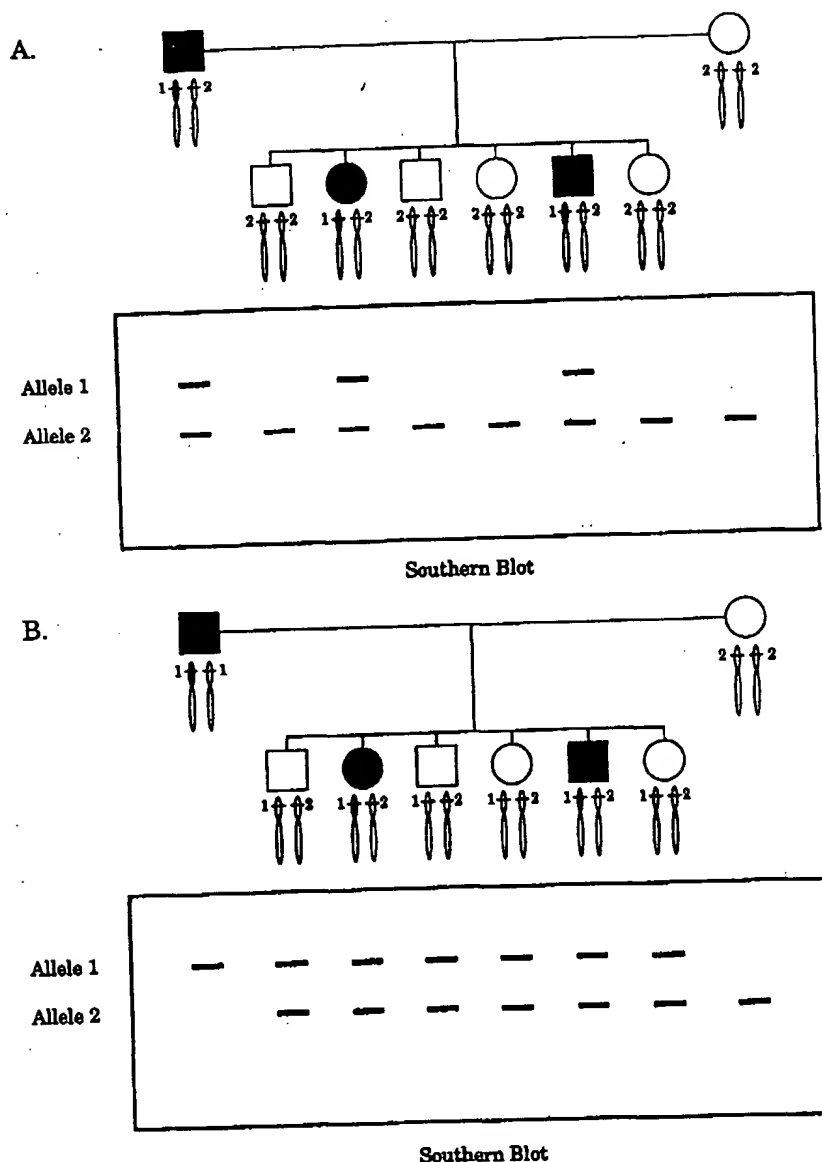
FIGURE 4.   *Example of a DNA mark-er that is linked to an autosomal dom-inant disease gene. In the family, indi-viduals who are thought to be phenotypically affected by a disorder are indicated by filled circles (females) or filled squares (males), whereas those who are thought to be normal are indicated by open circles or squares. The two polymorphic forms detected by a DNA marker of known chromo-somal location are indicated numeri-cally under each individual as 1 or 2. The arm of an idealized chromosome that contains a mutant gene causing the disorder is filled, and its normal homologue is open. The hypothetical appearance of these alleles on South-ern filters is indicated below each indi-vidual. In panel A, the affected parent is heterozygous for this hypothetical marker (a genotype of 1,2). If one of the alleles (in this case the 1 allele) cosegregates with the disease pheno-type more frequently than one would predict by chance, the disease gene and the DNA marker are likely to be linked. In panel B, on the other hand, the affected parent is homozygous at this DNA marker (a genotype of 1,1). In this case, linkage is not detectable even if it exists, because the genotype of each child will be identical, and cosegrega-tion cannot be established.*

linkage analysis. Because PCR-SSCP and PCR-DGGE can be used to examine many different DNA samples simultaneously, these techniques have been particularly useful in screening for disease-associated mutations.

Genetic Recombination

Linkage analysis would be simple (albeit much less powerful) if there were no recombination; in the ab-sence of genetic recombination, only 22 markers would be required to determine the autosomal location of a genetic locus. However, parental chromosomes are not transmitted to offspring in their original form; during the generation of germ cells, recombination, or crossing over, occurs between loci on homologous chromosomes (Figure 6). Genetic recombination events that occur during the first phase of meiosis (meiotic recombina-tion) occur with a frequency roughly proportional to the distance between the loci. Thus, two genetic loci that are separated by a few thousand base pairs would rarely recombine. Conversely, two loci on the same chromo-some that are separated by 20 million base pairs would recombine frequently, probably in approximately 20% of meioses. Two loci on the same chromosome that are so far apart that they recombine in 50% of meioses will appear to be unlinked, as the same pattern of coinher-itance (50%) would appear if the markers were located on separate chromosomes. From these data, the genetic distance between two linked loci can be estimated by calculating the extent of recombination that has oc-curred between them. In this way, ordered linkage maps of polymorphic DNA markers can be generated.

Because of recombination, it may be necessary to test hundreds of different polymorphic markers with differ-ent chromosomal locations before linkage is found, and even then the identification of linkage is not guaranteed. For example, if a disease gene is located on a chromo-somal segment that carries no defined markers, linkage would not be obtained. Thus, recombination has com-plicated linkage analysis in families and necessitated the development of many genetic markers. Once linkage
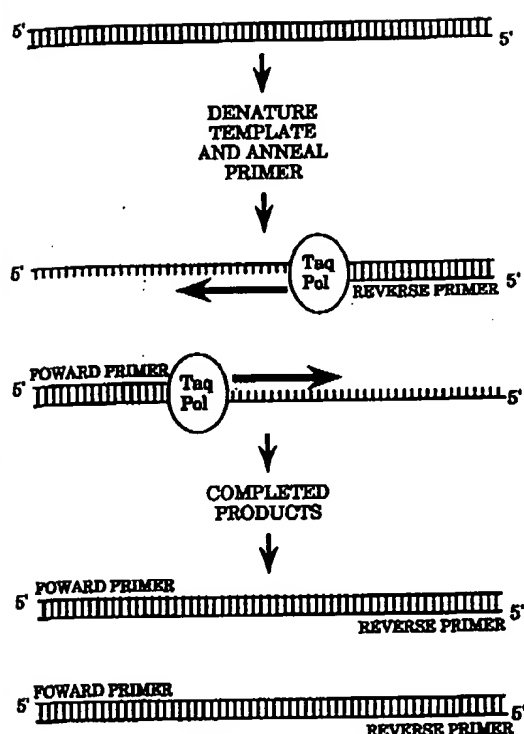
FIGURE 5. *Schematic of polymerase chain reaction (PCR), a method for copying DNA. Synthetic oligonucleotide primers complementary to specific sequences that flank a locus of interest are incubated with template DNA, which in this case might be human genomic DNA. If the reaction is first heated to 94°C and then cooled, the template DNA and oligonucleotide primer pair will denature and then reanneal. The addition of a primer-dependent polymerase (Taq pol) and nucleotide triphosphates to the reaction mixture results in the generation of two new strands of DNA that are complementary to the template. As a result, the template DNA has been duplicated. Cycles of PCR can be repeated to generate multiple copies of the DNA of interest.*

between a polymorphic marker and a disease locus has been identified, however, the identification of recombination between the disease locus and additional flanking markers is a powerful method for refining the location of the disease locus.

Linkage Maps of DNA Markers

Technical advances in molecular biology have facilitated the development of extensive banks of DNA-based polymorphic markers. Like genes, these markers have specific and consistent chromosomal locations. By linkage analysis, the position of one marker relative to other markers can be determined. In this way, linkage maps of genes and DNA markers have been generated for every human chromosome.[22,23] An example of a linkage map for markers on chromosome 11 is shown in Figure 7.

The task of generating a complete linkage map of the human genome is far from completion; by examining Figure 7, for example, one can see that large gaps in the map for chromosome 11 exist. One goal of the Human Genome Project is to refine linkage maps so that

continuous maps exist with markers separated by no more than 1 or 2 million base pairs. Nevertheless, the currently available DNA markers and linkage maps have greatly facilitated linkage studies involving human genetic disorders, problems that appeared to be unapproachable only a few years ago.

Linkage Analysis in Practice

Recently, we undertook a linkage study that may serve as an example of the usefulness of this approach. The long QT (LQT) syndrome is an inherited disorder that causes syncope and sudden death from cardiac arrhythmias, particularly malignant arrhythmias like torsade de pointes and ventricular fibrillation.[25-31] Presymptomatic diagnosis of LQT has been based on the identification of a prolonged QT interval on ECG, a finding that is often associated with this disorder. Unfortunately, this diagnostic test is imprecise, and many cases are not detected until symptoms arise. This problem is particularly unfortunate because treatment options exist for LQT.

It is reasonable to propose that improved diagnosis and treatment of LQT might result from a better understanding of the mechanisms that underlie this disorder. Unfortunately, we know very little about the pathogenesis of LQT. Mutations in sodium, potassium, chloride, or calcium channel genes or in genes that regulate these genes or gene products could lead to delayed myocardial repolarization in affected patients. This, in turn, could cause secondary depolarizations (afterdepolarizations) and predisposition to ventricular arrhythmias. There is little evidence, however, to support or refute any of these hypotheses, and testing each of them would be a formidable task, particularly because many of these genes have not been identified.[32]

LQT appears to be inherited as an autosomal dominant trait, so it is reasonable to hypothesize that this disorder is caused by the inheritance of a single gene. This hypothesis can be tested by linkage analysis. An advantage of this approach is that no knowledge of the biochemistry or physiology of the disorder is required for success.

Families

The first step in linkage analysis is to identify a suitable family. We were fortunate in having access to the largest single family with LQT that has been described (Figure 8). This family has been followed and carefully characterized for many years by Michael Vincent and Katherine Timothy of the LDS Hospital in Salt Lake City, Utah.[33-37] The story of this family begins in the mid 1800s, when the two brothers shown in the second generation of Figure 8 emigrated from Denmark to a small town in Utah. We do not know much about their health except that they were excluded from the family profession of fishing for health reasons. Nevertheless, they fathered the offspring shown in Figure 8. As one can see in the figure, the LQT phenotype is inherited as an autosomal dominant trait; every generation contains affected individuals, offspring of affected parents are affected approximately 50% of the time, and the sexes are approximately equally represented. This pattern of inheritance suggests that the LQT phenotype
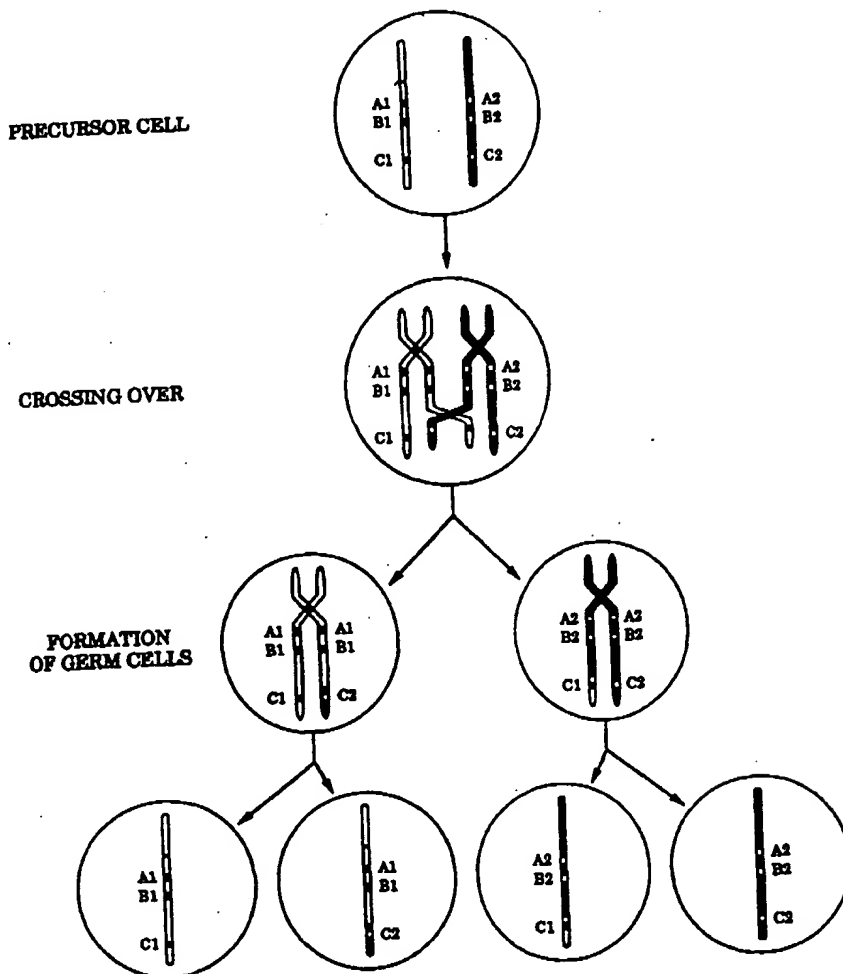
PRECURSOR CELL

CROSSING OVER

FORMATION
OF GERM CELLS

FIGURE 6. *Schematic of genetic re-combination. During the development of germ cells (meiosis), homologous chromosomes pair, and recombination occurs. In the precursor cells shown at top, the homologous chromosomes carry different alleles of three markers (A, B, and C). In the first phase of meiosis, the chromosomes are repli-cated. Sister chromosomes then pair, and homologous sections of DNA may be exchanged (cross over). A second cell division results in germ cells with half the normal complement of chro-mosomes. Some of the chromosomes are identical to those found in the precursor cell, whereas others are re-combinant. Recombinant chromo-somes are those containing unfilled and stippled segments derived from both parental chromosomes.*

in this family results from the inheritance of a single gene.

Phenotypic Characterization

The observable properties, such as the physical appearance, of an organism is said to be its phenotype. By contrast, the genetic constitution of an organism is said to be its genotype. The phenotype of an organism is produced by its genotype in conjunction with the environment. The second step in linkage analysis is to accurately determine the phenotype with respect to LQT of every individual in the family. As linkage analysis is based on a comparison of genotype and phenotype, phenotypic misclassifications may lead to false conclusions about linkage, so this step is an important one. Figure 9 shows the spectrum of cor-rected QT interval (QT_c; Reference 38) for family members at risk for inheriting the LQT gene. As one can see, two peaks exist, one with a mean that approx-imates the mean for a control group shown at the bottom of Figure 9. The second peak is much longer, with a mean of about 0.48 second. It is apparent, however, that these two curves overlap. To solve this problem, we chose a conservative approach to pheno-typing. Asymptomatic individuals with a QT_c of 0.41 second or less were classified as normal, and symptom-atic individuals with a QT_c of 0.45 second or greater and

asymptomatic individuals with a QT_c of 0.47 second or greater were classified as affected.[39] All family members that did not meet these diagnostic criteria were classi-fied as uncertain. As a result, the family was divided into three approximately equal groups.

Genotypic Characterization

The third step in linkage analysis is genotyping, a process that is outlined in Figure 3. DNA extracted from the leukocytes of each family member is incubated with a restriction enzyme. The resultant restriction fragments are separated by agarose gel electrophoresis and transferred to nylon filters. These filters are then incubated with a DNA marker that detects RFLPs in the general population. The pattern of RFLPs for each individual in the study is entered into a computer individual in the study is entered into a computer relational data base that interfaces with the LINKAGE programs.[40–43] These programs analyze the phenotypes and genotypes of individuals in the family and calculate the odds that a DNA marker and the disease gene are linked. The odds are presented in a logarithmic form called the LOD score (LOD is an acronym for the logarithm of the odds). A LOD score of +3 (odds of 1,000/1) is considered to be good evidence for linkage. A LOD score of −2 (odds of 1/100) effectively excludes linkage at a given locus.
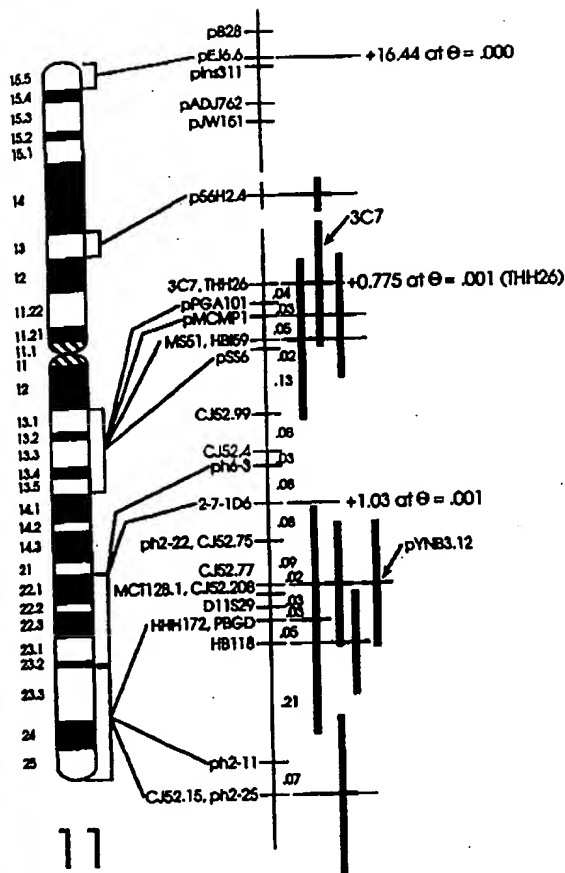
FIGURE 7. *Linkage map for DNA markers on chromosome 11. Long QT syndrome (LQT) is linked to Harvey ras-1 on chromosome 11p. A karyogram of chromosome 11 with the relative location of different chromosomal segments is shown at left. The relative location of polymorphic DNA markers is shown at right.[24] The recombination fraction between two loci is indicated. For example, DNA marker HB118 on the long arm of this chromosome (chromosome 11q 23.3) recombine at a frequency of approximately 21% (0.21) with DNA marker ph2-11. Regions of chromosome 11 that were excluded for linkage to LQT on the basis of a negative LOD score of less than −2 are indicated by the solid vertical bars at the extreme right. Linkage between LQT and markers at the Harvey ras-1 locus (pEJ6.6) was identified on the short arm of chromosome 11 (chromosome 11p15.5) as indicated by a LOD score of +16.44.*

Before linkage analysis can begin in full, the existence of misinheritances in the family must be excluded. As false paternity occurs with a fairly high frequency in the general population, this is an important step. The best DNA markers for establishing inheritance in a pedigree are those that are highly polymorphic and thus likely to be informative. As mentioned earlier, one group of very highly polymorphic markers detects VNTRs. It is not unusual for VNTR markers to detect more than 10 polymorphisms in the general population. An example of a misinheritance detected by a VNTR marker is shown in Figure 10.

Once we had resolved all misinheritances in the family, we were able to begin general linkage analysis.

As we had few other physiological clues and no data about the chromosomal location of the LQT locus, we began our study with highly polymorphic markers on chromosome 1 and worked our way to chromosome 22. In the process, LOD scores of more than +2 (odds favoring linkage of more than 100/1) were identified with two different markers. Although tantalizing, these scores proved to be the result of chance, because the scores did not improve when adjacent markers were tested. As linkage was not identified in our first series of experiments, we began to test additional markers from untested chromosomal regions until linkage was finally identified (Figure 7). In the process, we successfully scored more than 245 markers and excluded approximately 60% of the human genome. Linkage was identified using the Harvey ras-1 (H-ras-1) gene as a marker[38]; this marker detects a VNTR polymorphism located on the short arm of chromosome 11 (chromosome 11p; References 44 and 45). The LOD score was +16.43, indicating that the odds favoring linkage were greater than $10^{16}/1$.

Genetic Distance

As mentioned above, genetic recombination complicates linkage analysis and certainly increased the complexity of our study. Recombination frequencies, however, can be converted to genetic map distances using specific mapping functions that take into account undetected double-crossover events. For example, if recombination between two loci occurs with a frequency of 1%, the loci are said to be separated by a genetic distance of 1 centimorgan (cM). If, on the other hand, the recombination frequency is 20%, the map distance is approximately 26 cM (the relation between map distance and recombination fraction is not linear at high recombination frequencies because of the frequency of double recombinants). On average, 1 cM corresponds to a physical distance of about 1 million base pairs of DNA. In our study, no recombination was observed between the disease locus and H-ras-1. Thus, the maximum LOD score (Table 1) was identified at a recombination fraction of zero.[39] If, however, the data showed recombination between the two loci, the computer would have calculated a maximum LOD score at a specific recombination fraction, or theta (θ). As no recombination was observed between LQT and H-ras-1, these loci are tightly linked in genetic terms and are likely to be in close physical approximation (probably within 3 million to 6 million base pairs).

On the basis of the complete linkage between H-ras-1 and LQT, this proto-oncogene became a candidate gene for the disorder. This hypothesis was especially exciting because a physiological rationale for the involvement of ras in LQT exists. Recent experiments done in a cell-free system have shown that ras proteins and GAP, the GTPase activating protein, can regulate atrial potassium channels.[46] As abnormal myocardial potassium currents could cause LQT, it was not difficult to imagine that nononcogenic mutations in H-ras-1 might cause this disorder. Further evidence that a candidate gene like H-ras-1 is the disease gene can be obtained by identifying mutations in sequences derived from affected individuals. To date, no mutations have been identified in the coding sequence of the H-ras-1 gene in LQT pa-
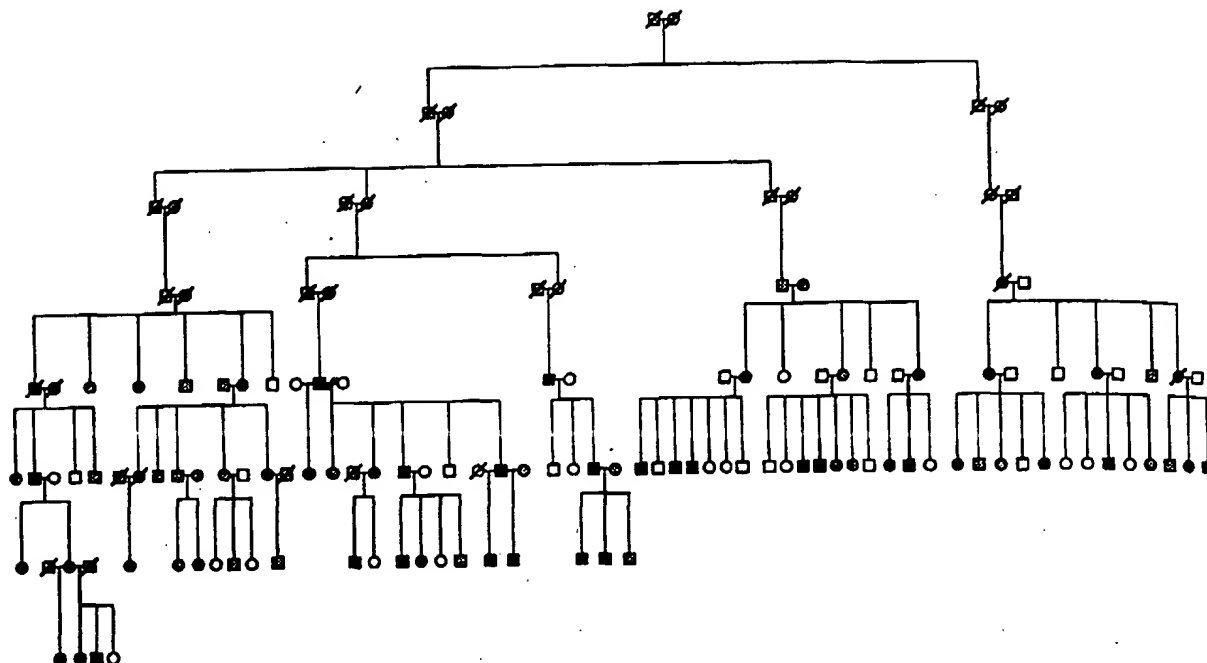
FIGURE 8.   Long QT syndrome (LQT) kindred 1532. Individuals having the characteristic symptoms of fainting or sudden death due to ventricular arrhythmias, with prolongation of the QT interval on ECG, are represented by filled circles or squares. Unaffected individuals are represented as empty circles and squares; individuals with an equivocal or unknown phenotype are stippled. The pedigree structure has been altered to protect confidentiality. Informed consent was obtained from each individual before the study began. In this family, LQT is inherited as an autosomal dominant trait.

tients (unpublished observations). On the basis of these data, we cannot be definitive about the candidacy of H-ras-1 in LQT. As will be described in more detail below, many other candidate genes for LQT are likely to reside in this chromosomal subunit, and each must be considered until the disease gene is identified.

Locus Homogeneity

As a result of these linkage studies, genetic diagnosis of LQT for members of the family shown in Figure 8 is secure. Can these markers be used to diagnose LQT in other families? In the six additional and unrelated LQT families studied to date, the answer is yes.[47] One of these secondary families is shown in Figure 11. The genotypes for each individual using the H-ras-1 gene as a VNTR marker are also shown. In the families studied, nine alleles were observed at this locus, and they are numbered 1–9. Focusing on the lower left section of the pedigree in Figure 11, one can see that the unaffected father is homozygous at the H-ras-1 locus with a 4,4 genotype. The phenotypically affected mother, on the other hand, is heterozygous at ras with alleles 3 and 8. All of the children should inherit the 4 allele from their father and either the 3 or the 8 allele from the affected mother. As one can see, all the affected children inherited the 8 allele from their mother, whereas normal children inherited the 3 allele. In the rest of the family, every affected individual inherited the 8 allele, indicating that in this family, like the original family studied, LQT is probably linked to loci on the short arm of chromosome 11. The combined LOD score for the six additional families was 5.25, indicating that the odds favoring linkage were greater than 10⁵/1. Again, the

maximum LOD score was at a recombination fraction of zero, indicating that no recombinants would exist in any of these families. Thus, the H-ras-1 marker can be used in these families for presymptomatic diagnosis of LQT.

Limitations of Linkage Analysis

Although linkage analysis using recombinant DNA technology is quite powerful, it has limitations. It is possible, for example, that mutations at several different genetic loci cause a phenotype that is similar to LQT. Locus heterogeneity for other disorders, such as hypertrophic cardiomyopathy[48–50] and the Marfan syndrome,[51] has been described, and it would not be surprising to learn that LQT is similar. In that event, markers on chromosome 11p15.5 would not be useful for genotypic diagnosis in unlinked families.

Even if the majority of LQT proves to be a result of mutations in one gene, linkage analysis cannot be used to determine the gene-carrier status of individuals in very small families or in sporadic cases. In Figure 11, linkage between the marker and the disease gene was suggested because the disease phenotype always cosegregated with a specific genotype (the 8 allele) in this large family. Multiple meioses were scored in several generations, and marker informativeness was good (affected parents were heterozygous). In a smaller family (Figure 12A) or in a sporadic case (Figure 12B), it may not be possible to establish linkage, even if the marker is highly polymorphic and very tightly linked to the disorder. In the small family shown in Figure 12A, identification of linkage is not possible because there are not enough meiotic events available for a statistically significant LOD score to be established. In the
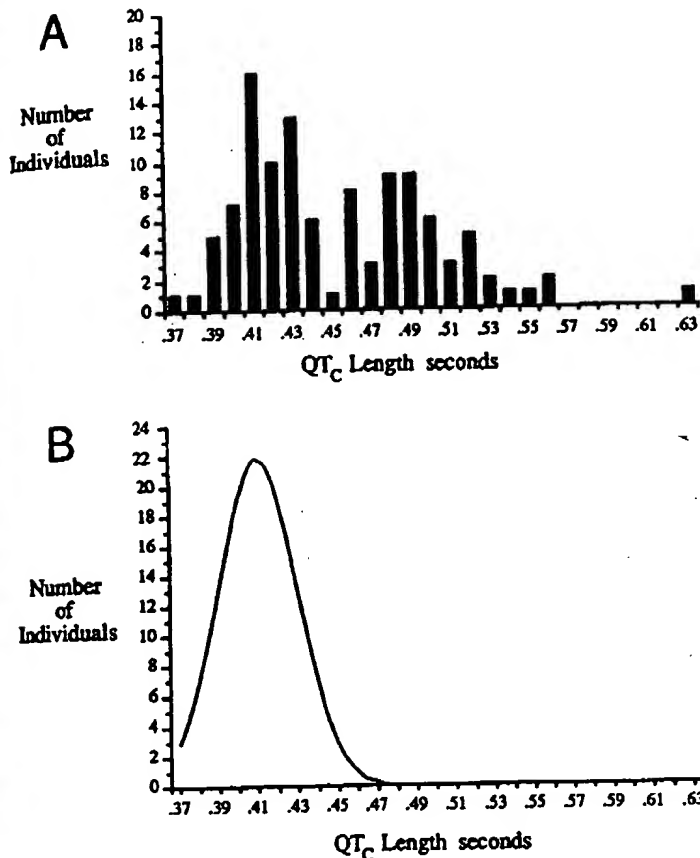
FIGURE 9. *Graphs showing distribution of corrected QT (QT$_c$) intervals for long QT syndrome (LQT) kindred 1532 (panel A) and for a control group (panel B). The number of individuals with a given QT$_c$ is indicated on the Y axis, and the actual QT$_c$ interval in seconds is indicated on the X axis.*

sporadic case shown in Figure 12B, on the other hand, no meiotic event can be scored because neither parent is known to be phenotypically affected. The only exception to this limitation occurs when the genetic variation that causes the disorder is detectable as a RFLP or other polymorphism. For example, the mutation that causes sickle cell anemia causes a specific restriction enzyme site change that is detectable as a RFLP. The polymorphism detected by the H-*ras*-1 gene is a VNTR and is not the cause of LQT, so improved diagnosis of LQT in sporadic cases will not be available until we have identified and characterized the mutations that cause this disorder. Sporadic cases of LQT may be a tremendous help, however, because they may lead us to
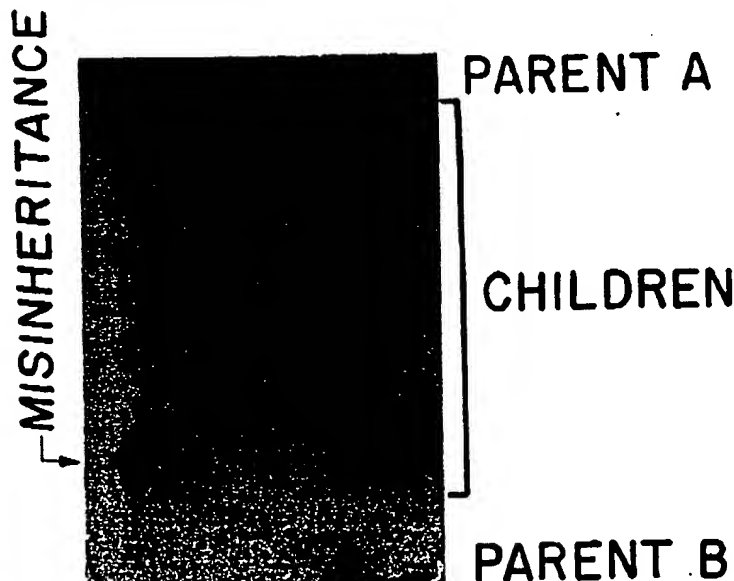

FIGURE 10. *Variable number of tandem repeats (VNTR) marker shows misinheritances. Genotypes are shown for individuals in a family by use of a VNTR marker located on chromosome 2. The genotypes for the children are flanked by genotypes for the mother at left and for the father at right. Each child inherited one allele from the mother and one allele from the father. One child (arrow) fails to inherit an allele from the father. If this pattern is confirmed after resampling and repeated testing using multiple markers, it is suggestive of false paternity, and this child would not be included in a linkage study.*

TABLE 1. LOD Scores (Pairwise) Between Long QT Syndrome and a DNA Marker (pUCEJ6.6) in a Five-Generation Pedigree

Recombination fraction	0.00	0.05	0.10	0.20	0.30	0.40
LOD scores	16.44	15.18	13.81	10.79	7.42	3.76

Linkage analysis was performed with the computer program LINKAGE. A DNA marker defining a restriction site polymorphism at the H-*ras*-1 locus, pUCEJ6.6, was found to be tightly linked to the long QT syndrome (LQT) locus, with a maximum LOD score of 16.44 at a recombination fraction of zero. This score corresponds to odds of >10^{16}/1 in favor of linkage. LOD scores have been calculated with penetrance of 0.90. As no estimates of LQT gene frequency exist, for purposes of this study the frequency of this rare disease gene was assumed to be 0.001. LOD, logarithm of the odds.

new, easily detectable, mutations in the LQT gene, thereby defining that gene. For that reason, we are eager to examine these individuals.

Identification of Disease Genes by Mapping and Mutational Analysis

The obvious concern that one has when initiating a linkage study is that linkage will not be identified. As DNA markers and linkage maps continue to improve, such concerns will be minimized, and continued improvement will be limited by the availability of families and phenotyping difficulties. Once linkage between a DNA marker of known chromosomal location and an inherited disease has been clearly identified, the focus turns to identification of the disease gene, and a number of strategies become possible. With luck, a gene that was previously mapped to the same chromosomal segment will become a candidate for the disease gene because of its chromosomal location and the feasibility of its involvement in the disorder. Frequently, however, no reasonable candidate gene is apparent, and a different, much more labor-intensive approach must be taken. In general, primary linkage analysis localizes a disease gene to a DNA segment of approximately 4 million to 10 million bases on either side of the linked marker. This DNA segment may contain hundreds of genes, most of which have yet to be identified and characterized. To identify the disease gene in such a large sea of nucleotides can be daunting, but it can be done. The general strategy that can be used has been referred to as "reverse genetics" or "positional cloning," but we generally use the phrase "mapping and mutational analysis" because it more clearly defines the process. The first step is to confine the disease gene to a chromosomal segment of about 1 million base pairs. This can be done by a combination of refined linkage and physical mapping. To improve the linkage map, one must identify new polymorphic markers that are very tightly linked to, but are recombinant with, the disease phenotype in families. These markers are called flanking markers and can be identified from cloned DNA segments from the region of interest. As the goal is to identify linked markers that recombine with the disease phenotype, it is very helpful to identify and characterize new extended families with the disorder. Another localizing method that has proved very helpful for disease gene identification is the identification of chromosomal anomalies like translocation and small deletions that are associated with the disease. If such an anomaly exists in
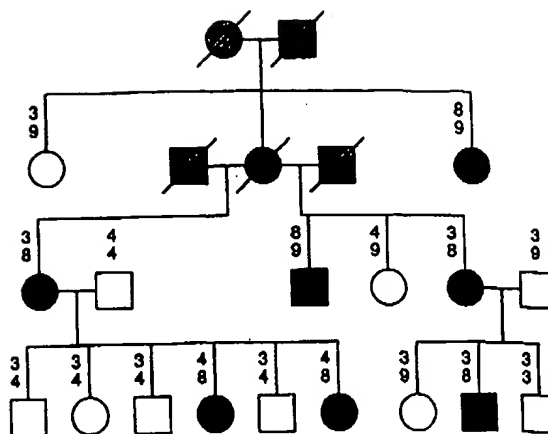

FIGURE 11. *Long QT syndrome (LQT) pedigree showing H-ras-1 genotypes. Individuals having the characteristic pattern of fainting or sudden death due to ventricular arrhythmias, with prolongation of the QT interval on ECG, are represented by filled circles or squares. Above each symbol, individual alleles are listed for the marker pUC EJ 6.6 (Harvey ras-1). At this variable number of tandem repeats marker locus, the restriction enzyme Msp I revealed nine distinct alleles within the families, ranging in size from 1 to 5.1 kilobases (kb). The disease gene cosegregates with the 1.2-kb allele (No. 8) in this kindred. The pedigree structure has been altered to protect confidentiality.*

a patient's DNA, its identification may help localize the disease gene to a segment of several hundred bases, greatly facilitating the process.

Once the disease gene has been confined between flanking markers to a chromosomal segment of about 1 million base pairs, the identification of genes that will be candidates for the disease gene solely on the basis of their location becomes feasible. First, the DNA that
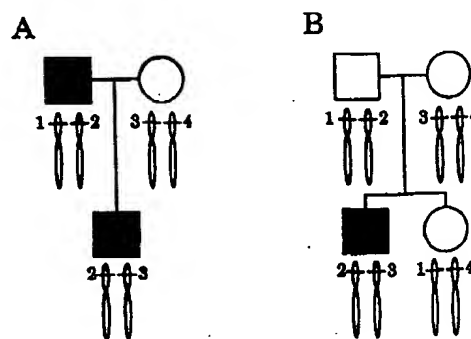

FIGURE 12. *Pedigrees showing limitations of linkage analysis. Linkage analysis is not informative for genetic diagnosis in small families (panel A) or in sporadic cases (panel B) because a pattern of phenotype/genotype cosegregation cannot be established. Individuals thought to be affected by a disorder are indicated by filled squares, and individuals thought to be phenotypically normal are indicated by empty circles or squares. The mutant gene causing the disease is indicated by a filled chromosomal segment. Genotypes for an adjacent variable number of tandem repeats marker are indicated numerically.*

spans the flanking markers must be isolated and cloned in overlapping segments of 30,000–400,000 bases. DNA markers from these cloned segments must be developed so that the orientation of the overlapping clones can be determined. This process of physical mapping, sometimes called chromosome walking, is laborious, but improvements in the size and specificity of genomic DNA libraries and technical advances continue to facilitate the process. Next, the genes that reside in the physically mapped DNA segments must be identified, generally by using genomic DNA clones to screen complementary DNA libraries. Any complementary DNA that maps back to the correct chromosomal segment immediately becomes a candidate for the disease gene and must be characterized. At this point, the goal is to identify disease-associated mutations in the candidate gene, a process that may involve many different techniques, often including PCR-SSCP or PCR-DGGE and direct sequencing. Evidence that a candidate gene is the disease gene includes the cosegregation of a mutation with the disease in one family, the identification of mutations in the same gene in different families, and the identification of germ line mutations in sporadic cases of the disease. Once the gene has been identified, the structure and function of the protein product of the gene can be examined by use of biochemical and physiological techniques. These studies may support the hypothesis that specific mutations are important in disease pathogenesis and should facilitate insight into the molecular mechanisms of the disease. The process of disease gene identification by mapping and mutational analysis is challenging, but the ends justify the effort.

Summary

Linkage analysis can be used to determine the chromosomal location of a disease gene in a setting in which little or no physiological information is available. Recombinant DNA technologies have greatly facilitated the development of new, highly polymorphic DNA markers, which, in turn, have made general linkage analysis feasible in humans. Most of our progress has been in studies of disorders that are caused primarily by the inheritance of one gene, so-called single-gene disorders, like LQT, which we have linked to chromosome 11p15.5. However, better mapping techniques and detailed genetic maps also offer hope for an improved understanding of the genetic variations that are important in complex disorders like hypertension and atherosclerosis.

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